EFFECTS OF POTENTIAL SIGNAL TRANSDUCTION ANTAGONISTS ON PHYTOALEXIN ACCUMULATION IN TOBACCO

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; EGTA; W7; calmodulin; calcium-dependent protein kinase; calcium uptake; sesquiterpene; elicitation; magnesium; verapamil; lanthanum; staurosporine; A23187; cadmium.

Abstract—Tobacco cell suspensions produce sesquiterpene phytoalexins when treated with cellulase. This response is enhanced by $CaCl_2$, $MgCl_2$ or $CdCl_2$. For $CaCl_2$ and $MgCl_2$, enhancement extends for 8 hr after elicitor treatment. Several classes of signal transduction antagonists were tested on this plant system. EGTA inhibits sesquiterpene accumulation with an effective concentration range suggesting that Ca^{2+} is the divalent cation being affected. Mg^{2+} is as effective as Ca^{2+} in relieving the inhibition by EGTA. This type of shared role by Ca^{2+} and Mg^{2+} has not been described previously and is yet to be characterized. The timing of the EGTA effect suggests a role of Ca^{2+} in signal transduction. Calcium ion influx across the plasma membrane is one possibility since inhibitors of Ca^{2+} influx inhibit sesquiterpene accumulation induced by cellulase. Verapamil is an inhibitor and its action is relieved by the Ca^{2+} ionophore A23187. Among the cations which inhibit Ca^{2+} influx, La^{3+} is the most effective inhibitor of sesquiterpene accumulation. Calmodulin, or a Ca^{2+}-dependent protein kinase, is also implicated in the signal transduction sequence, based on the results with W7, W5 and staurosporine. Neomycin and LiCl, inhibitors of phosphoinositide cycle steps, enhance cellulase-elicited sesquiterpene accumulation, suggesting a complex interaction of this cycle in the phytoalexin response. The results indicate that Ca^{2+} and Mg^{2+} play several roles in sesquiterpene biosynthesis. The involvement of a multicomponent elicitor signal transduction pathway, with calcium ion functioning at more than one step, is suggested.

INTRODUCTION

Tobacco cell suspensions produce sesquiterpene phytoalexins (capsidiol, phytuberin, phytuberol and debneyol) when inoculated with pathogens such as *Pseudomonas solanacearum* or *Phytophthora infestans* or when treated with cellulase or fungal glucans [1–3]. Phytoalexins are synthesized by biosynthetic pathways that are activated in response to elicitor treatment. Evidence that calcium ion influx has a role in the induction of sesquiterpene phytoalexin accumulation in potato tuber slices has been presented [4], and calcium ion influx also appears to be involved in isoflavonoid phytoalexin induction in soybean cell suspensions [5], but not in pea tissues [6–8] or French bean cell suspensions [9]. Calcium ion influx is involved in triggering the hypersensitive response-associated potassium ion/proton exchange in tobacco cell suspensions inoculated with *Pseudomonas syringae* pv *syringae* [10]. We are interested in the signal transduction event(s) triggered by elicitors of sesquiterpene biosynthesis in tobacco cell suspensions. The purpose of the current study was to investigate whether or not calcium ion influx has a role in elicitation (as measured by sesquiterpene accumulation) and to investigate possible involvement of later steps in the calcium-mediated signal transduction process.

RESULTS AND DISCUSSION

Treatment of tobacco cell suspensions with cellulase induces the synthesis and accumulation of the sesquiterpene phytoalexins, capsidiol, debneyol and phytuberol (Fig. 1). The cellulase preparation used in these studies (Onozuka RS, from *Trichoderma viride*) was previously reported to elicit phytoalexins in tobacco suspension cells [11]. According to the distributor (Karlan Chemical Co., Santa Rosa, CA), the enzyme preparation contains cellulase activity (16 000 U g^{-1}) and some xylanase activity. This commercial enzyme preparation successfully elicits phytoalexins at very low concentrations (0.1 $\mu g\,ml^{-1}$), and we now have evidence that the actual eliciting component is an endoxylanase activity (data not shown). Capsidiol and debneyol accumulations are detectable 4 hr after treatment with the cellulase preparation and

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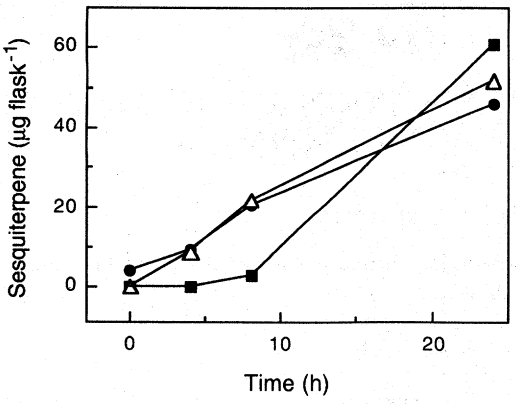

Fig. 1. Extracellular levels of sesquiterpene phytoalexins after treatment of tobacco cell suspensions with cellulase. Cellulase was added at 0 hr. Capsidiol (△), debneyol (●), phytuberol (■). In controls (no cellulase) cells at 8 hr, 1.0 μg flask^{-1} of debneyol and 1 μg flask^{-1} of capsidiol were detected. At 24 hr, the levels were 1.9 μg flask^{-1} debneyol and no detectable capsidiol. No phytuberol was detected in control cells at any time.

values at 24 hr are about five times higher than those at 4 hr. Since phytuberol is not detected until the 24 hr sampling time, in most studies only capsidiol and debneyol are reported. A cellulase preparation which has been boiled does not elicit sesquiterpene accumulation at 24 hr when used at concentrations up to 1 μg ml^{-1}.

Extracellular calcium and magnesium

Addition of 1 mM $CaCl_2$ to cell suspensions strongly enhances cellulase-elicited sesquiterpene accumulation (Table 1), while $CaCl_2$ has no effect on sesquiterpene accumulation of unelicited cells (data not shown). Thus although extracellular $CaCl_2$ is not an elicitor by itself, it clearly plays a role in the amount of sesquiterpene phytoalexin accumulating in the culture filtrate.

Magnesium chloride also strongly stimulates the levels of extracellular sesquiterpenes (Table 1). As is observed with $CaCl_2$, $MgCl_2$ is not an elicitor by itself. Although Mg^{2+} has not been implicated in signal transduction sequences, it is an important cofactor in ATP synthesis and reductive processes, and may have been limiting in this regard. Whereas 1 mM $CaCl_2$ or $MgCl_2$ enhances sesquiterpene accumulation, 10 mM $CaCl_2$ or $MgCl_2$ is slightly inhibitory, probably having reached physiologically toxic levels.

The divalent cation chelator, EGTA [ethyleneglycol-bis-(β-aminoethylether) N,N,N′,N′-tetraacetic acid], which is commonly used to probe for Ca^{2+} function, inhibited cellulase-elicited accumulation of capsidiol and debneyol (Fig. 2). The effect on capsidiol and debneyol was apparent at both 8 and 24 hr after treatment. The concentration of EGTA needed to inhibit sesquiterpene accumulation by 50% was between 2 and 5 mM. EGTA has the ability to chelate many divalent cations which are present in the culture media, including Cu^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+}. With the exception of Mg^{2+}—for which it has unusually low (10-fold less) affinity—the affinity of EGTA for all of the other cations is greater than its affinity for Ca^{2+} [12]. Thus, the free concentrations of these cations in the presence of EGTA were calculated. The concentration of free Mg^{2+} at 10 mM EGTA is still 97% of that in the absence of EGTA. The concentrations of free Ca^{2+} are 2.9 mM (97.7% of the total Ca^{2+}), 2.12 mM (70.7%), 1.08 mM (36%), 65 μM (2.2%) and 18 μM (0.6%) in the presence of 0.1, 1, 2, 5 and 10 mM EGTA, respectively. With cellular Ca^{2+} concentrations in the low micromolar range, it appears that 5–10 mM EGTA would be needed to affect processes mediated by Ca^{2+} influx; this is observed for sesquiterpene elicitation (Fig. 2). Among the other cations

Table 1. The effects of $CaCl_2$, $MgCl_2$ and EGTA on capsidiol accumulation elicited by cellulase in tobacco cell suspensions

Treatment*	Contribution of added chloride salts (mM) Ca	Mg	Total cation concentration (mM) Ca	Mg	Free cation concentration‡ (mM) Ca	Mg	Capsidiol Relative amount ± s.e.†
H_2O			3	1.5	3	1.5	100 ± 6
$CaCl_2$	1		4	1.5	4	1.5	142 ± 10
	10		13	1.5	13	1.5	83 ± 9
$MgCl_2$		1	3	2.5	3	2.5	168 ± 17
		10	3	11.5	3	11.5	92 ± 13
EGTA			3	1.5	0.018	1.46	55 ± 12
EGTA + $CaCl_2$	10		13	1.5	3.13	1.50	138 ± 23
EGTA + $MgCl_2$		10	3	11.5	0.019	12.1	123 ± 26

*Final concentration of EGTA was 10 mM. EGTA was added before the cells; the elicitor, cellulase, was added after the cells; chloride salts were added after the elicitor. Culture filtrate was harvested 24 hr later.

†Values are expressed relative to the control (no EGTA or added salts), in which 25 ± 4 μg capsidiol accumulated per flask.

‡Free cation concentrations were calculated using 'Chelator' software.

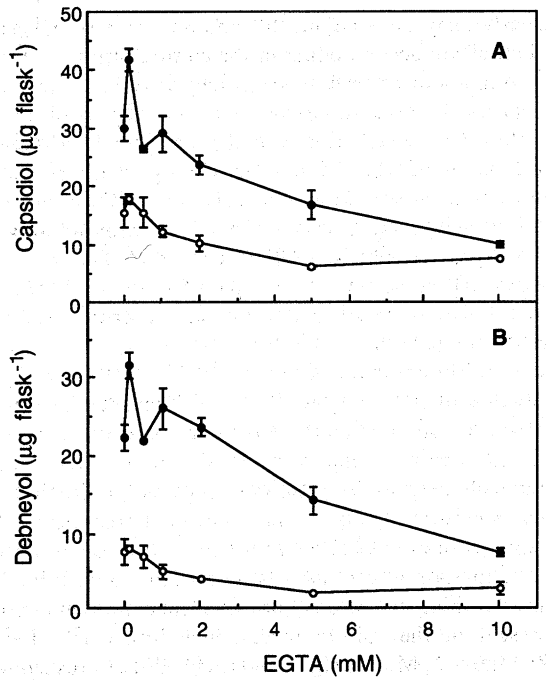

Fig. 2. Inhibition of sesquiterpene accumulation by increasing dose of EGTA in tobacco cell suspensions 8 (○) and 24 (●) hr after treatment with cellulase. EGTA was added approximately 30 min before the cellulase. (A) Capsidiol, (B) debneyol. Vertical bars indicate ±s.d.

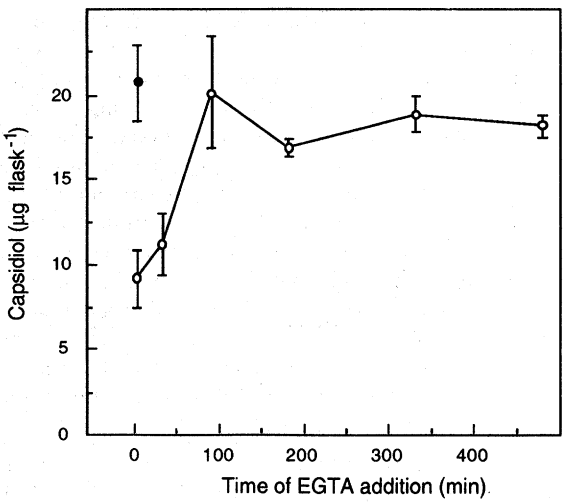

Fig. 3. The effect of time of EGTA addition on its inhibition of cellulase-elicited capsidiol accumulation in tobacco cell suspensions. Control (cellulase but no EGTA) (●); EGTA at 10 mM (○). Cellulase was added at 0 min and capsidiol accumulation was determined 24 hr later. Vertical bars indicate ±s.d.

present, free Cu^{2+}, Fe^{2+}, and Zn^{2+} are reduced to 0.0008, 23 and 4% of their total concentrations by 0.1 mM, 1 mM and 1 mM EGTA, respectively. Since the physiological concentrations of these cations are not known, their role in phytoalexin elicitation can only be deduced from the shape of the chelation curve, which is much steeper than that for Ca^{2+} and out of the effective range of EGTA concentrations.

Low levels of EGTA (0.1 mM) enhance capsidiol and debneyol accumulation up to 30% over the control (Fig. 2). This enhancement remains unexplained at present, although EDTA at 0.1 mM was equally effective in the observed enhancement. An inhibitory effect on sesquiterpene accumulation by one of the divalent cations present at 0.1 mM or less may be indicated by this result. Treatment with EDTA or EGTA (at 0.1 mM) alone does not result in detectable sesquiterpene accumulation.

EGTA (10 mM) does not inhibit elicitation if added 90 min or later after treatment with cellulase (Fig. 3), indicating that the effect of EGTA as an inhibitor of capsidiol accumulation occurs early in the elicitation process, and suggesting signal transduction. Lack of inhibition by the later treatments with EGTA also rules out a secondary toxic effect of EGTA on tobacco cell metabolism.

Addition of an equimolar amount of $CaCl_2$ restores free Ca^{2+} to about 3 mM, and relieves the inhibition of sesquiterpene accumulation by EGTA (Table 1). We were unable to rule out a nonspecific effect of divalent cations using $MgCl_2$ as is customarily done, since $MgCl_2$ is a strong enhancer by itself. $MgCl_2$ appears to relieve the inhibition of sesquiterpene accumulation by EGTA. This effect of $MgCl_2$ is not interpreted to be an additive effect, since similar concentrations in the absence of EGTA are inhibitory. Also, the 10 mM $MgCl_2$ only restores 1 μM of free Ca^{2+}. Thus it appears that free Ca^{2+} has a role in signal transduction for which $MgCl_2$ can substitute. This role for $MgCl_2$ has been observed in the presence of EGTA and at $MgCl_2$ concentrations that are normally inhibitory.

We attempted to distinguish the enhancing effects of $MgCl_2$ and $CaCl_2$ by a time course experiment, in which each was added at increasing times after cellulase treatment, expecting that added $CaCl_2$ (in the absence of EGTA) would enhance sesquiterpene accumulation only during the 60–90 min period of susceptibility to EGTA, while the effectiveness of $MgCl_2$ might be of longer duration. Enhancement by addition of $MgCl_2$ up to 8 hr after cellulase treatment was observed (data not shown). However, its effect is not clearly distinguished from the $CaCl_2$ treatment in which enhancement by $CaCl_2$ added at 3 and 8 hr was sometimes observed.

These experiments with Ca^{2+} and Mg^{2+} suggest more than one role for the extracellular cations in elicitation of sesquiterpene accumulation. First, it appears that concentrations in the media are not optimum; 25–20% greater concentrations of either of the cations resulted in significantly enhanced sesquiterpene levels. These enhancing effects of Ca^{2+} or Mg^{2+} extend through 8 hr after addition of elicitor, and thus probably are not related to the signal transduction events. Second, the inhibition by EGTA most likely results from reducing the free Ca^{2+} level to, or slightly below, the cellular level. The early effect of EGTA suggests that the Ca^{2+} has a role in signal

transduction. The involvement of Mg^{2+} in this second role needs further characterization.

One possibility of a signal transduction-related role for Ca^{2+} for which Mg^{2+} can substitute is the induction of localized defence responses based on the binding of Ca^{2+} or Mg^{2+} to pectic components [13]. This extracellular role for Ca^{2+} has been suggested to exist in tomatoes [14] based on the observation that the minimum degree of polymerization of oligogalacturonides required for activity as elicitors of localized defence responses coincides with the minimum degree of polymerization reported for the oligogalacturonides to form intermolecular complexes with calcium ions. Magnesium ions also bind to oligogalacturonides, albeit with three- to five-fold lower affinity than calcium ions [13].

Calcium ion influx

Three cations (which had been shown to inhibit Ca^{2+} influx in tobacco cell suspensions [10]) were tested. In the presence of $LaCl_3$, $CoCl_2$ or $CdCl_2$ (each at 500 μM), accumulation of capsidiol relative to the control is 36, 72 and 125%, respectively (Table 2). A correlation is observed between the relative effectiveness in inhibiting Ca^{2+} influx [10] and in inhibiting capsidiol accumulation. $LaCl_3$ is the most effective of the three cations both in inhibiting Ca^{2+} influx and capsidiol accumulation. We observe 50–70% inhibition of sesquiterpene accumulation 24 hr after treatment with cellulase plus $CoCl_2$, however $CdCl_2$ (at concentrations as low as 50 μM), enhances phytoalexin accumulation. The enhancement by Cd^{2+} might be reflecting an independent elicitor activity, such as that reported in *Datura stramonium* hairy root cultures [15].

Consistent with the hypothesis that Ca^{2+} influx stimulates phytoalexin accumulation, both nifedipine and verapamil, blockers of voltage-gated calcium channels in mammalian systems [16], inhibit the cellulase-induced accumulation of capsidiol and debneyol (Table 3). Verapamil is less effective than nifedipine, however verapamil is the only blocker whose effect appears to be restricted to inhibition of Ca^{2+} influx alone: the divalent cation ionophore A23187 does not completely relieve the inhibition by nifedipine, although it does nearly relieve the inhibition by verapamil. A23187 does not enhance cellulase elicitor activity by itself within experimental error. Since A23187 should be able to relieve the blockage of Ca^{2+} influx by both verapamil and nifedipine, these results suggest that nifedipine is not acting simply as a channel blocker. This interpretation of the data is consistent with previous reports using other plant tissues. Although both nifedipine and verapamil are inhibitors of voltage-gated calcium channels in animals, only verapamil, of the phenylalkylamine class, is an accepted Ca^{2+} channel blocker in higher plant cells [17] such as carrots [18, 19], zucchini [20] and maize [21]. Nifedipine, of the 1,4-dihydropyridine class, was tested without effect in each of these plants, although in the present study inhibition was observed.

Inhibition of sesquiterpene accumulation is proportional to the concentration of $LaCl_3$ (Fig. 4) or verapamil. Significant inhibition is observed in the micromolar range for $LaCl_3$, and for verapamil and nifedipine (not shown) as well. Two hundred micromolar is high relative to K_is reported for *in vitro* assays (e.g. [18]). Pharmacological side effects are always a concern [16]. On the other hand, higher levels of inhibitors might be expected to be needed in our system, given the high levels of divalent cations in the medium. In studying cytokinin-induced bud formation in the moss *Funaria*, Saunders and Helper observed that the higher the concentration of Ca^{2+} in the external medium, the higher the concentration of $LaCl_3$, vera-

Table 2. Effects of La^{3+}, Cd^{2+}, Co^{2+} on cellulase-elicited capsidiol, debneyol, and phytuberol accumulation in the culture filtrate

	Capsidiol	Deb + Pol
Treatment*	Relative value ± s.e.†	
H_2O	100	100
$LaCl_3$	36 ± 7	32 ± 5
$CoCl_2$	72 ± 24	49 ± 11
$CdCl_2$	125 ± 15	113 ± 14

*Chloride salt (500 μM final concentration) was added to flasks, followed by cell suspension (10 ml). After swirling to mix salts with cells, cellulase (50 μl) was added. Culture filtrate was collected 24 hr after treatment with elicitor.

†Cellulase-treated control accumulated 32 ± 12 μg capsidiol, 65 ± 14 μg debneyol (Deb) plus phytuberol (Pol) combined.

Table 3. Effects of the Ca^{2+} influx antagonists, nefidipine and verapamil, and the divalent cation ionophore, A23187, on capsidiol and debneyol accumulation elicited by cellulase

	Capsidiol	Debneyol
Treatment*	μg ± s.d.	
Nefidipine	10 ± 0.1	53 ± 6
Nefidipine + A23187	16 ± 2	58 ± 4
Verapamil	17 ± 5	31 ± 16
Verapamil + A23187	31 ± 5	71 ± 1
Control†	39 ± 7	72 ± 16
A23187	43 ± 4	86 ± 7

*Inhibitor concentration was 200 μM; A23187 concentration was 10 μM. Inhibitor and/or A23187 (in 100 μl ethanol–dimethyl formamide 9:1 v:v) was added to flasks, followed by cell suspension (10 ml). After swirling to mix inhibitor with cells, cellulase (50 μl) was added immediately. Culture filtrate was collected 8 hr after treatment with elicitor.

†Cellulase-treated control, treated with 100 μl ethanol–dimethyl formamide 9:1 v:v.

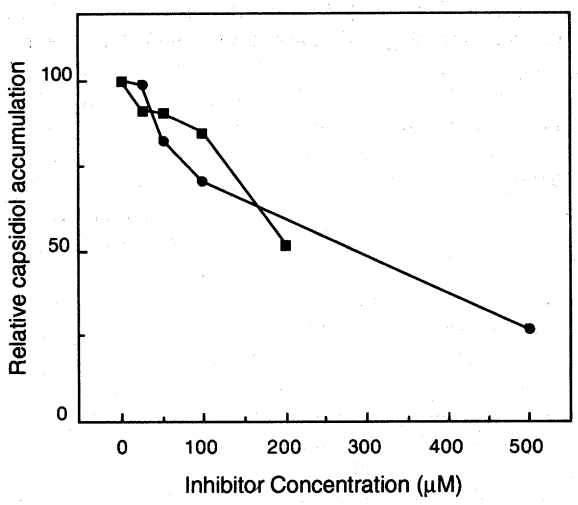

Fig. 4. Inhibition by W7 (■) and lanthanum (●) of capsidiol accumulation by tobacco cell suspensions 24 hr after treatment with cellulase.

pamil or methoxyverapamil (D600) that was required to evoke 50% inhibition [22].

A23187 alone (in the absence of cellulase) does not elicit phytoalexin accumulation (data not shown), hence Ca^{2+} influx alone is not sufficient to signal the phytoalexin response in tobacco cell suspensions. In contrast to this lack of an observed effect in tobacco cell suspensions, A23187 by itself is sufficient to elicit accumulation of glyceollin by soybean cell suspensions [5].

The results with lanthanides and verapamil suggest a role for Ca^{2+} influx in sesquiterpene phytoalexin elicitation in tobacco cell suspensions, in agreement with similar studies reported with potato tuber slices [4] and soybean cell suspensions [5]. In contrast, it would appear that Ca^{2+} influx is not a signal transduction component of isoflavonoid phytoalexin induction in peas [6–8] or French beans [9].

Other evidence for cellulase-induced changes in membrane permeability

We have recently reported that treatment of tobacco suspension cells with cellulase (at the same concentrations which elicit phytoalexins in the current study), also causes a rapid fatty acylation of sterol lipids [23]. Since sterols can comprise up to 50 mol% of the plasma membrane lipids, and are thought to be localized mainly, if not exclusively, in the plasma membrane, it is conceivable that such changes in lipid composition may affect membrane permeability. Indeed, we have recently found that $LaCl_3$ (which inhibited cellulase-induced phytoalexin production by about 60% in the current study), also inhibited the cellulase-induced fatty acylation of sterols by about 50% (Moreau and Powell, unpublished data). Further work is required to determine whether the cellulase-induced elicitation of phytoalexins is related to the cellulase-induced shift in sterols, but the fact that both processes are inhibited by $LaCl_3$ provides evidence that they may be linked in some way and may be indicative of a change in membrane permeability.

Calcium ion interaction with a calmodulin-like factor

The calmodulin antagonist, W7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide], inhibits capsidiol accumulation in cellulase-treated tobacco cell suspensions, with effective concentrations in the micromolar range (Fig. 4). W7, but not its inactive analogue W5 [N-(6-aminohexyl)-1-naphthalenesulphonamide], inhibits sesquiterpene accumulation by 40% at 0.2 mM (Table 4). The specificity of W7 effectiveness suggested that a Ca^{2+}–calmodulin interaction is involved. This might be due to the involvement of free calmodulin, Ca^{2+}-dependent protein kinase [24, 25] containing a calmodulin-like domain [25], or protein kinase C (also Ca^{2+}-dependent and inhibited by other calmodulin antagonists [26]). Staurosporine, a known inhibitor of protein kinase C [27], inhibits accumulation by nearly 100% when tested at 2.5 μM, a strong indication of a role for protein kinase C-like activity in the signal transduction pathway (Table 4).

Recently, Vogeli *et al.* [28] reported that phytoalexin biosynthesis in tobacco suspension cells was inhibited by calcium/calmodulin antagonists. Using fungal glucan as an elicitor, they reported that capsidiol production, and sesquiterpene cyclase induction were both inhibited by W7, but not by W5. In our hands, using cellulase as an elicitor, effects similar to those after fungal elicitation were observed when the cells were treated with W5 or W7.

Other signal transduction systems

Two inhibitors of the phosphoinositide cycle steps in animal systems, neomycin (a phospholipase C inhibitor), and LiCl (an inositol-1-phosphatase inhibitor [29]) enhance cellulase-elicited sesquiterpene accumulation in tobacco cell suspensions (Table 4). These results suggest a complex interaction of the phosphoinositide pathway, which also regulates intracellular Ca^{2+} flux, in the phytoalexin response. It is currently believed that if phosphoinositides are involved in signal transduction in higher plants, their characteristics are quite different from the animal model [30].

Tyrosine protein kinase activity has been reported in plants under the conditions of virus infection [31]. Inhibitors of this activity, genistein (Table 4) and quercetin (not shown) both inhibit cellulase-elicited sesquiterpene accumulation by 90% at 200 $\mu g\,ml^{-1}$. However, the structurally-related compound, daidzein, which is inactive as a tyrosine protein kinase inhibitor [32], is equally effective (Table 4). Thus, these results suggest that the mode of inhibition of sesquiterpene accumulation by these compounds is not through an effect on tyrosine protein kinase activity.

Table 4. Effects of various calmodulin and protein kinase inhibitors, and inhibitors of the phosphoinositide cycle on cellulase-elicited capsidiol and debneyol accumulation

Treatment*	Concentration	Capsidiol	Debneyol
		μg±s.d.	
Control (methyl sulphoxide)		24±2	14±0.5
W7	200 μM	13±3	9±2
W5	200 μM	22±1	14±0.4
Staurosporine	2.5 μM	nd†	1±1
Genistein	20 μg ml^{-1}	25±0.1	14±0.4
	100	14±6	9±4
	200	1±0.3	nd
Daidzein	20 μg ml^{-1}	26±9	16±3
	100	2±0.2	1±0.2
	200	1±0.01	0.2±0.3
Neomycin	100 μM	25±1	15±2
Control (H_2O)		23±4	16±2
LiCl	50 μM	27±1	23±2
	100	28±2	23±1
	500	30±2	24±0.1

*Inhibitor was added to flasks, followed by cell suspension (10 ml). After swirling to mix inhibitor with cells, cellulase (50 μl) was added immediately. Culture filtrate was collected 8 hr after treatment with elicitor.

†nd, None detected.

Conclusions

More than one role for $CaCl_2$ and $MgCl_2$ in cellulase-elicited, extracellular sesquiterpene accumulation in tobacco cell suspensions is indicated. First, an enhancing effect of either salt is observed which extends through 8 hr after elicitation and is thus not related to signal transduction. Second, an early role for Ca^{2+} is indicated by the effective concentration range and timing of the observed inhibition by EGTA. Magnesium chloride is able to relieve this inhibition without restoring free Ca^{2+} concentrations, indicating a role for Ca^{2+} for which Mg^{2+} can substitute. This role for Ca^{2+}/Mg^{2+} needs further characterization. Third, another role of Ca^{2+} consistent with the EGTA experiments could be Ca^{2+} influx, as supported by the results with the Ca^{2+} channel blockers, verapamil and $LaCl_3$. Fourth, probing later steps in the calcium-mediated signal transduction process, a protein kinase C-like activity also appears to be involved in the phytoalexin response, based on the specificity of the effect of W7 and on strong inhibition by the protein kinase C inhibitor, staurosporine. In summary, these results suggest that at least two steps of the 'calcium-mediated signal transduction process' are involved in the induction of phytoalexins and other stress metabolites, in particular the requirement for Ca^{2+} influx, and the further involvement of calmodulin and/or a Ca^{2+}/calmodulin-dependent or C-type protein kinase. In addition, enhancement of cellulase-elicited sesquiterpene accumulation by neomycin and LiCl suggested involvement of the phosphoinositide pathway. Finally, it should be recognized that a possible mechanism of action (inhibition) of some of the inhibitors studied could involve direct interaction with the cellulase preparation, thus preventing it from acting or thus preventing its action as an elicitor. The reduced levels of phytoalexins caused by some of these chemical inhibitors could also be due to their either decreasing permeability of the plasma membrane or preventing active secretion of phytoalexins (since only the levels of extracellular phytoalexins were measured in this study). Our recent studies of cellulase-induced changes in plasma membrane lipid composition [23] and our observation that La^{3+} inhibits this process, provide further support for this hypothesis.

EXPERIMENTAL

Nifedipine and the calcium ionophore, A23187, were purchased from Calbiochem, methyl sulphoxide was from Aldrich, and cellulase (Onozuka RS) was obtained from Karlan Chemical Company, Santa Rosa, CA. Other reagents were purchased from Sigma. Tobacco cell suspension cultures were the generous gift of Dr Joseph Chappell, University of Kentucky.

Tobacco cell suspension cultures were maintained as described previously [33]. Briefly, cultures were maintained at 25° on a rotating shaker in Murashige and Skoog salt mixt. (Gibco Cat #500-1117) supplemented with 2,4-dichlorophenoxyacetic acid (0.5 mg l^{-1}), sucrose (30 g l^{-1}), thiamine–HCl (10 mg l^{-1}), nicotinic acid (1 mg l^{-1}), pyridoxine–HCl (1 mg l^{-1}) and myo-inositol (100 mg l^{-1}). Concns of selected salts were: $CuSO_4$, 1 μM; $CoCl_2$, 1 μM; $ZnSO_4$, 30 μM; $FeSO_4$, 100 μM; $MnSO_4$, 100 μM; $MgSO_4$, 1.5 mM and $CaCl_2$, 3 mM. Cultures were transferred every 7 days by diluting with 4 vols fresh

media and aliquoting. Three-day-old cultures were used for experiments. Cell suspensions (10 ml in 25 ml Erlenmeyer flasks) were treated with the elicitor (cellulase at 0.1 μg protein ml^{-1}, final concn) and test compounds were added in the order indicated in each experiment, and incubated for up to 24 hr at 25° on a rotating shaker. The concns of free divalent cations were calculated using the "Chelator" program (C. Patton, Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950, U.S.A.), for pH 5.9, 25° and 0.045 M ionic strength. Treatments were duplicated within each experiment and each experiment was repeated 2–4 times. At the indicated times after treatment, culture filtrates were collected through Miracloth on a Buchner funnel with mild vacuum. Sesquiterpenes were extracted from the medium with two vols of Et_2O. Quantitative analysis of the phytoalexins was performed via HPLC equipped with a flame ionization detector and a Spherisorb CN (5 micron, 3 × 100 mm) column under isocratic conditions with C_6H_{12}–*iso*PrOH (97:3, v:v) [34]. The retention times of the phytoalexins were 3.3, 3.9 and 11.9 min for phytuberol, debneyol and capsidiol, respectively. Values presented are means within experiment ± standard deviation (s.d.) or means between experiments ± standard error (s.e.).

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